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<b>(54) Title:</b> THIOL ACTIVATION OF CYTOTOXIC AGENTS AND ROOT FORMATION STIMULATION  <b>(57) Abstract</b>  Thiol compounds are employed in conjunction with compounds which modulate cell proliferation, both as to plants and mammals. The thiol compounds find effect in enhancing the effectiveness of auxin like compounds, either as herbicides or in enhancing root formation. In addition, thiol compounds act in conjunction with cytotoxic agents in mammals to allow for lower dosages of the cytotoxic agents while retaining the cytotoxic effect against hyperproliferative diseases, such as cancer. The combination finds particular effect against cells dependent upon an isoform of a surface membrane NADH oxidase for proliferation.		

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## THIOL ACTIVATION OF CYTOXIC AGENTS AND ROOT FORMATION STIMULATION

### INTRODUCTION

#### Technical Field

5           The field of this invention is the killing of proliferating cells and enhancing root formation.

#### Background

          There are many situations where one wishes to modulate cellular growth. Since all of life is dependent upon cellular regeneration, there is substantial interest  
10   in being able to modulate cellular growth in the plant and animal kingdoms. In the case of plants, one wishes to encourage the growth of desirable plants, such as plant crops and flowers, while at the same time one wishes to discourage the growth of weeds, both pre-germination and post-germination of the desired plant. In many situations one wishes to kill all vegetation, such as highway borders, pathways,  
15   graveled areas, and the like. However, many of the herbicides which are used today pollute the environment in introducing undesirable elements into the soil, which can then be leached into aquifers, streams and rivers. There is, therefore, substantial interest in being able to reduce the amount of herbicidal use without further pollution of the environment.

20           There is also the need to root stock to expand desired plants, particularly woody stock. Better methods are needed to increase the efficiency and reduce the time for rooting.

          Finally, there is the proliferation of cells in the animal kingdom. The development of the various species from a single fertilized cell to the mature adult  
25   requires rapid proliferation. However, during the life of the organism, there are many situations where rapid proliferation is undesirable. Various diseases associated

with rapid proliferation include cancers, psoriasis, restenosis, and the like. Many of the agents used for treating these diseases, are cytotoxic agents which attack all proliferating cells and frequently have substantial side effects in addition to their cytotoxic activity.

5 Various techniques have been employed to reduce the systemic influences of these cytotoxic drugs. Immunotoxins employ a toxin joined to an antibody, where the antibody is specific to varying degrees for a marker present on cancer cells. Another technique which is limited to physical access to the tumor employs the cytotoxic drug in a matrix which reduces the translocation of the cytotoxic drug  
10 from the site of the lesion. Other techniques have involved use of liposomes with agents, such as antibodies, to direct the liposomes to the target, where the lumen of the liposome comprises a cytotoxic agent. However, none of these techniques has found universal acceptance. So long as one continues to use the cytotoxic drugs in systemic fashion, there is substantial interest in finding ways to diminish the amount  
15 required for an effective dosage as a cytotoxic agent.

#### Relevant Literature

The classic method of root induction is to utilize an auxin shock. Lane (1978) Plant Sci. Lett. 13:281-285 and Moncousin (1992) Rooting of *in vitro* Cuttings. In: Biotechnology in Agriculture and Forestry (YPS Bajag, ed.) Springer  
20 Verlag, Heidelberg, 17:231-261. Standardi and Romani (1990) Hort. Sci. 25:1435-1436 teaches auxin shock for rooting and employs cysteine.

### SUMMARY OF THE INVENTION

Thiol compounds are employed as adjunctive agents to enhance modulation  
25 of cellular proliferation. Particularly, the thiol agents are found to enhance cytotoxicity, both in plants and animals, as well as enhancing root formation in conjunction with auxins or auxin shock.

### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

30 Methods and compositions are provided for modulation of cellular proliferation in plants and animals. Particularly, the thiol compounds are used in conjunction with cytotoxic agents to reduce the amount of the cytotoxic agent

required for cytotoxicity. By combining the thiol compound with the cytotoxic agent, either locally or systemically, substantially smaller amounts of cytotoxic agent are required in order to achieve desired levels of cytotoxicity. In mammals, one may use combinations of cytotoxic agents plus thiol compound to control the growth  
5 of cells in proliferative diseases, such as cancer. The combination may be administered systemically or locally.

#### CYTOTOXICITY OF PLANT CELLS

The control of undesired vegetation will be considered first. The subject compositions comprise an auxin-type herbicide, e.g. 2,4-dichlorophenoxyacetic acid  
10 and trichlopyr, optionally in conjunction with ammonium nitrate, and the thiol compound. The auxin type herbicide may be used as an acid derivative, particularly salts and esters, where the ester group may be of from 1 to 12 carbon atoms and the salts may include amino salts of from 0 to 12 carbon atoms. These derivatives are well known in the literature and commercially available.

15 A wide variety of thiol compounds may be employed, the primary consideration will be economics, convenience, effect on the environment, ease of formulation, handling characteristics, physiological activity toward mammals, and the like. A number of thiol compounds are available and may be used, such as cysteine, glutathione, dithiothreitol, thiolpropionic acid, etc. For the most part, the  
20 compounds will generally be from about 2 to 12, usually 2 to 10 carbon atoms, and may have other functionalities, such as oxy, oxo, including esters and amides, amino, etc. As indicated above, the choice of mercaptan is not critical to this invention, so long as it is not too volatile, does not have an offensive odor, and can be readily handled.

25 Depending upon the particular auxin-type herbicide employed, one will normally use less than about 60%, usually less than about 50%, and generally more than about 20% of the dosage necessary for herbicidal activity of the auxin-type herbicide, by itself, or in conjunction with ammonium nitrate. The amount employed will vary depending upon the particular vegetation involved, the degree to  
30 which one wishes to kill the vegetation and the rapidity with which the vegetation is to be killed. Thus, where it is sufficient that only a portion of the wild vegetation be killed, milder formulations may be employed, where rapid complete death is

desired, the formulations will be stronger.

- Generally, the amount of herbicide will vary with the particular herbicide, generally ranging from about 1 to 6 pounds per acre, preferably from about 1 to 5 pounds per acre, more preferably from about 2 to 4 pounds per acre. When
- 5 ammonium nitrate is present, it will generally range from about 0.025 to 2 pounds per acre, more usually from about 0.05 to 1.5 pound per acre. The thiol compound will vary to some degree based on the particular thiol compound but will generally be in the range of about 0.01 to 2 pounds per acre, more usually in the range of about 0.025 to 1.5 pounds per acre, and preferably 0.05 to 1 pound per acre.
- 10 Formulations of particular interest for total vegetative kill include 3 to 5 pounds per acre of the herbicide, particularly trichlopyr, 1 to 2 pounds per acre of ammonium nitrate, and 0.05 to 0.25 pounds per acre of cysteine.

- The subject formulations can be formulated in a variety of ways, such as dispersible dry powders, pellets, solutions, or the like. In the formulation, the
- 15 weight ratio of thiol compound to herbicide will be generally in the range of about 1:2-100, more usually in the range of about 1:10-100, while the ratio of the ammonium nitrate to herbicide will generally be in the range of about 1:1.5-100 more usually 1:2-50.

- Other additives and excipients may be included in the formulation, depending
- 20 upon the manner in which the formulation will be applied. Other additives which may be included in minor amounts include spreading agents, penetrating agents, surfactants, emulsifiers, etc., with the other components in appropriate relationship.

- The subject compositions can be used pre-emergent, and may find application post-emergent, particularly as a spray, although pellets may also be used and
- 25 scattered. For the spray, the herbicide will generally be about 1 to 10, more usually 2 to 8, preferably 3 to 6 pounds per 100 gallons, the ammonium nitrate if present, will generally be 0.5-5, usually about 2 to 4, pounds for 100 gallons and the thiol compound will generally be about 0.1 to 1, more usually about 0.1 to 0.5 pounds for 100 gallons. The composition may be provided as a concentrate, for further
- 30 dilution, where the herbicide will be from about 1 to 5 pounds per gallon, usually employing an emulsifier.

In some instances, combinations of herbicides may be employed, such as

combinations of triclopyr, picloram, 2,4-D, glyphosate, clopyralid, fanoxaprop-ethyl, oxadiazon, bromoxynil, propanil, dicamba, and the like, where the additional herbicide may provide specific advantages for particular applications. Generally, the weight ratio of the secondary herbicide(s) to the primary auxin type herbicide

5 described above, will generally be from about 0.01-0.5:1, more usually from about 0.05-0.25:1.

When spraying, the application rate will generally provide the amount of herbicidal composition indicated above. Conveniently, one may provide for formulations which allow for spraying at about 10 to 50 gallons per acre, more

10 usually at about 15 to 35 gallons per acre.

The subject formulations are particularly applicable to woody trees and brush, where the species are resistant to the auxin-type herbicide, by itself or in conjunction with other herbicides. The subject compositions are found to be effective for non-evergreen roadside vegetation and deciduous wood species, such as

15 ash and hard maple.

Species that are controllable and very sensitive to the herbicides include briars (blackberry, raspberry, etc.), mulberry, walnut, sumac, sassafras, box elder, buckeye, cottonwood, wild grape and poison ivy; species that are sensitive include red bud, elm, honey locust, black locust, hackberry, willow, and white oak; species

20 of intermediate sensitivity include hard maple and black cherry; and species that are resistant include juniper and ash, all of which can be controlled by the subject formulation.

#### CYTOTOXICITY OF MAMMALIAN CELLS

The thiol compounds act via alterations in the activity patterns of a growth-related NADH oxidase (disulfide-thiol interchange protein). This activity is altered

25 in cancer and a protein bearing this altered activity circulates in the blood. The circulating form of the cancer associated altered form of the NADH oxidase has been designated as t-NOX and is useful as a diagnostic procedure for cancer. The thiol compounds find use as an enhancer of in conjunction with diagnostic uses of

30 drugs acting as inhibitors of t-NOX. With patient sera treated with 1  $\mu$ M to 100  $\mu$ M capsaicin, the maximum inhibition of NADH oxidation with serum is 15 to 40%. However, if the capsaicin treatment is followed by 0.1 to 10  $\mu$ M reduced

glutathione, a pronounced additional transient inhibition of 5 to 10 min duration is observed. This inhibition is not observed with sera of normal patients.

The thiol compounds can also find use for reactivation of sample preparations that have been frozen prior to use in a diagnostic assay for cancer associated t-NOX. Addition of 0.1 to 10  $\mu$ M of oxidized glutathione to such preparations restores the enzymatic activity. Inclusion of up to an equivalent amount of reduced glutathione with the serum samples during storage will also help maintain enzyme activity. Comparable amounts of oxidized thiol compounds other than oxidized glutathione also find use.

For use in mammals to inhibit cellular hyperproliferation particularly in association with cancer, various cytotoxic agents may be used in conjunction with thiol compounds. The subject compositions may be used with any type of cancer, whether solid or dispersed, including carcinomas, sarcomas, lymphomas, melanomas, and the like. The cancers may be associated with various tissues and organs, such as blood, breast, prostate, ovary, lung, kidney, liver, eye, skin, pancreas, bone, bone marrow, leukocytes, etc.

Chemotherapeutic drugs which may be employed include enzyme inhibitors, proliferation inhibitors, lytic agents, DNA synthesis inhibitors, membrane permeability modifiers, DNA intercalators, antimetabolites, or the like. Illustrative drugs include chlorambucil, melphalan, busulfan, carmustine, lomustine, streptozotocin, thiotepa, dacarbazine, methotrexate, 5-fluorouracil, cytarabine, azaribine, mercaptopurine, thioguanine, vinblastine, vincristine, actinomycin D, adriamycin, bleomycin, mithramycin, mitomycin C, L-asparaginase, cisplatin, sulfonylureas, capsaicin, and its derivatives, and the like. Of particular interest are drugs which inhibit a surface membrane NADH oxidase associated with the hyperproliferative state. These compounds include adriamycin, sulfonylureas, capsaicin, and the like.

The chemotherapeutic drug will normally be used in an amount of from about 10 to 80 weight percent, more usually from about 10 to 60 weight percent, particularly from about 20 to 60 weight percent of the normal chemotherapeutic dosage. The thiol compounds, depending upon the particular drug, may be formulated together with the chemotherapeutic drug or separately and combined



immediately prior to administration or concomitantly or consecutively with administration of the chemotherapeutic drug. The amount of the thiol compound which finds use in proportion to the amount of chemotherapeutic drug will generally be optimized within the ratio of about 0.01 to 10, more usually from about 0.05 to 1  
5 of thiol compound to chemotherapeutic drug. Thiol compounds of interest include cysteine, glutathione, thiolacetic acid, cysteine, 2-mercaptoethanol, dithiothreitol, mercaptamine, etc.. Since the thiol compounds will normally be physiologically acceptable and relatively large amounts of the thiol compound can be used without adverse physiological effect, once the minimum amount is defined for the desired  
10 therapeutic activity, one may use increasingly larger doses of the thiol compound without adverse effect and frequently without any further advantage.

The subject composition may be used both *in vitro* and *in vivo*. In many situations, it is desirable to inhibit growth of cancer cells in the presence of normal cells in culture. By augmenting the activity of the chemotherapeutic agent, with an  
15 agent such as the thiol compound which does not normally have adverse physiological effects, one can substantially reduce the adverse effect of the chemotherapeutic agent on the normal cells. In this manner, one can minimize the adverse effects on normal cells, while insuring the substantial absence or absence of tumorous cell growth. The chemotherapeutic agent and thiol additive may be  
20 maintained at a substantially constant concentration replenishing the culture medium on a regular basis, conveniently by substituting one half of the culture medium on a schedule of from 1 to 7 days.

For use in mammals, the subject compositions can be introduced intralesionally or parenterally, particularly intravascularly or intramuscularly. For  
25 intralesional administration, the subject compositions may be injected directly into the lesion, by themselves or in conjunction with vasoconstrictive agents, and/or matrices, such as collagen, fibrinogen, heparin, or the like, individually or in combination. See, particularly, U.S. Patent No. 4,619,913. Generally, the amount of drug will range from about 0.01 to 30% of the composition depending upon the  
30 nature of the drug. Usually, the amount of cytotoxic drug administered to the tumor site will range from about 0.01 to 500, more usually about 0.05 to 300 mg/kg of the host. As illustrative, about 50 to 200 $\mu$ M of a chemotherapeutic sulfonylurea

localized concentration is provided. The thiol concentration will therefore be in the range previously indicated in relation to the chemotherapeutic agent.

For intravascular administration, the formulation may be present in any convenient physiologically acceptable medium, to provide a systemic concentration of the chemotherapeutic agent in proportion to the normal dosage. Carriers may include water, saline, phosphate buffered saline, vegetable oils, ethanol, or the like. Concentrations may be varied widely, depending on the manner of administration, solubility of the chemotherapeutic agent, etc. Small amounts of other additives may be present, such as emulsifiers, stabilizers, buffers, and the like. In some instances, the subject formulation may be provided in the lumen of a liposome for slow or directed release.

#### ROOTING OF SHOOTS

Rooting is carried out in three phases. Phase 1 is induction (0 to 3 or 4 days.) Phase 2 is dedifferentiation and the formation of root meristems (1 to several weeks). phase 3 is growth and emergence of new roots (several weeks to 1 month). In some situations, especially with plantlets *in vitro*, the induction phase following the auxin shock is carried out in darkness (up to 5 days). Thereafter, rooting is either in continuous light or alternating light and dark cycles appropriate to the species. For stem cuttings, all 3 phases of rooting are carried out either in continuous light or under alternating dark and light cycles appropriate to the plant material.

In conjunction with rooting, thiols may be used as adjuvants to enhance root stimulation, particularly with difficult-to-root varieties. The thiol compounds described above, particularly cysteine and dithiothreitol, find use in this application in conjunction with auxins in the growth medium or auxin shock. Auxins include indole-3-acetic acid, indole-3-butyric acid,  $\alpha$ -naphthaleneacetic acid, etc. Prior to auxin shock, the cuttings or shoots may be subject to maintenance in the dark from 0.5 to 2 weeks under mild conditions. The auxin shock may be carried out as appropriate, generally employing from about 0.5 to 5 mM of an appropriate auxin, conveniently 1-2 mM indole-3-acetic acid (IAA). The auxin shock is performed in a conventional manner, conveniently using an agarized solution and placing the microcutting into the solution for from 0.5 to 5, more usually from about 0.5 to 3,

and preferably from 1 to 2 h. The timing may vary depending upon the particular species and its resistance to formation of roots. The microcuttings may be obtained from callus, tissue, intact plants or other appropriate plant parts or sources of growing tissues where formation of roots might be desirable. The auxin solution  
5 will be in a solvent comprising such other additives as minerals, vitamins, salts, buffers, detergents, stickers, wetting agents, spreaders and other growth enhancers.

Instead of auxin shock, a combination of auxin and thiol compound can be employed in the rooting medium. Various conventional rooting mediums may be employed such as Murashige and Skoog mineral solutions with conventional  
10 additives, such as inositol, thiamine, nicotinic acid, indolebutyric acid and a carbon source e.g. sucrose; a woody plant medium mineral solution (Lloyd and McCown, (1981) Int. Plant Prop. Soc. Proc. 30:421-427) supplemented with vitamins (Mullin et al. (1974) Phytopath. 64:1425-1429) and amino acids (Skoog (1944) Am. J. Bot. 31:19-24), with plant growth regulators, such as  
15 benzylaminopurine and alpha-naphthylacetic acids, with a carbon source and an amino acid source, such as Difco bactoagar. The amount of auxin in the rooting medium will be about 2 mM. The particular medium will be selected in accordance with conventional ways for the particular plant.

The thiol compound may be provided with the auxin shock or auxin  
20 continuously or intermittently during the first 10 days of maintenance in the shooting medium. After the auxin shock, the cuttings or shoots are rinsed and then introduced into an agar medium or distilled water comprising the appropriate composition. The thiol compound will generally be present in about 0.05 to 5 mM, preferably about 0.1 to 2 mM, where the response may vary with the particular thiol  
25 source and the time at which the thiol compound is provided. Usually, during growth, the thiol compound will be provided over at least one day and may be provided during the entire period, with some preference for the first one to three day on the six to nine day periods from the initiation or rooting stimulation with the auxin shock or auxin containing rooting medium.

30 A wide variety of monocots and dicots both non-woody and woody plants, may be used with the subject formulation. Illustrative plants include apple, dipladenia, daphne, mung bean, soybean, Hedera, Forsythia, Salix, various Prunus

species, Chrysanthemum, Poinsettia, Acacias, Acer, Alnus, Azalea, Bougainvillea, Bouvardia, Castanea, Carica, Chaelomenes, Cercis, Citrus, Cornus, Corylus, Cotinus, Eucalyptus, Cydonia, Fagus, Gardenia, Garrya, Ginko b., Hamamelis, Hydrangea, Ilex, Kalmia, Malus, Pieris, Pyrus, Quercus, Ribes, Rosa, Skimmia,  
 5 Syringa, Vaccinium, Viburnum, Vitis, Rubus, Spirae and various other fruit-bearing, ornamental and commercially-utilitarian woody species as well as flowers, vegetables and agricultural and horticultural crop species as well as conifers and evergreens.

The following examples are offered by way of illustration and not by way of  
 10 limitation.

### EXPERIMENTAL

#### I. Effect of Thiol Compounds on Activity of Herbicides

A number of studies were performed to evaluate the effect of various combinations of auxin like compounds, with and without ammonium nitrate, and  
 15 without a thiol additive and with different thiol additives on a variety of weeds and woody shrubs. These particular plant species are frequently found in areas where vegetation is undesired and there is a strong interest in maintaining the area substantially free of vegetation. The results of the studies are set forth in a series of tables, tables 1 to 9.

20

Table 1. Comparison of triclopyr (Garlon-4) and the ammonium nitrate additive combined with TR-III (Cysteine) on control of ash and hard maple. IN-126 test area. Treatments were applied August 10, 1993. Evaluations were on October 14, 1993.

Amount/40 gal water X-77			Defoliation, %		
Triclopyr	Ammonium nitrate	surfactant	TR-III	Ash	Hard Maple
2 lb	1 lb	0.1 gal*	None	60 ± 10	50 ± 10
2 lb	1 lb	0.1 gal	1 lb	90 ± 10	100 ± 0

30

\* 0.25% of the total spray mixture

Table 2. Effect of cysteine in combination with 2,4-D and trichlopyr on kill of white clover (*Trifolium repens*).

5	Herbicide	Cysteine 0.05 lb/A	% Dead			
			Applied 5/31/94, Evaluated 6/6/94	Applied 6/1/94, Evaluated 6/22/94	Applied 6/6/94, Evaluated 6/19/94	Applied 6/7/94, Evaluated 7/5/94
10	None	-	0	0	0	0
		+	0	0	0	0
	2,4-D, 5 lb/A	-	30	20	15	20
		+	50	40	30***	30***
	Trichlopyr, 5 lb/A	-	60	60	50	60
		+	80*	80***	70***	100***

\* 90% kill on 6/12/94 and 100% kill on 6/19/94.

\*\* 90% kill on 6/27/94.

\*\*\* Contained 1 lb/A ammonium nitrate.

20

Table 3. Comparison of cysteine and glutathione in combination with 2,4-D and trichlopyr on kill of white clover (*Trifolium repens*).

25	Herbicide	Cysteine	Glutathione	Dithiothreitol	% Dead	
					Applied 6/8/94, Evaluated 7/5/94	Applied 6/9/94, Evaluated 6/19/94
	2,4-D, 2 lb/A	-	-	-	20	20
		1 lb/A	-	-	30	30
30		-	1 lb/A	-	50	-
		-	-	1 lb/A	-	30
	Trichlopyr, 2 lb/A	-	-	-	90	40
		1 lb/A	-	-	100	50
35		-	1 lb/A	-	90	-
		-	-	1 lb/A	-	60

Table 4. Dose response of reduced glutathione, dithiothreitol and cysteine on herbicidal activity of 3.5 lb/A trichlopyr on white clover (*Trifolium repens*). Applied 6/30\*\*, 7/1/94\*\*\* and 7/6/94\*\*\*\*. Evaluated 7/20/94\*\*, \*\*\* and 7/23/94\*\*\*\*.

Rate/A	% Dead		
	Glutathione**	Dithiothreitol***	Cysteine****
0	5	20	50
0.05	90	40	60
0.1	100	50	50
0.1*	100	50	30
0.5	80	40	60
1	80	50	40

\*+0.1 lb/A ammonium nitrate

Table 5. Enhancement of herbicidal activity of 5 lb/A trichlopyr on white clover (*Trifolium repens*) by ammonium nitrate. Applied 6/16/94. Evaluated 6/26/94.

Ammonium nitrate, lb/A	% Dead
0	40
0.05	55

Table 6. Dose response of cysteine in the presence of 2 lb/A trichlopyr and 1 lb/A ammonium nitrate on white clover (*Trifolium repens*).

Cysteine Rate/A	% Dead			Average
	Applied 7/6/94 Evaluated 7/23/94		Applied 7/7/94 Evaluated 8/1/94	
0	80	50	70	67
0.025	100	80	90	90
0.05	80	60	90	77
0.1	90	60	80	77
0.5	100	60	80	80
1	100	80	80	87

Table 7. Control of common milkweed (*Asclepias syriaca*) and canada thistle (*Cirsium arvense*) by trichlopyr + ammonium nitrate + cysteine.

5	Rate/A, lb			% Dead		
	Trichlopyr	Ammonium Nitrate	Cysteine	Milkweed ( <i>Asclepias syriaca</i> )	Canada thistle ( <i>Cirsium arvense</i> )	Field Bindweed ( <i>Convolvulus arvensis</i> )
10	2	-	-	20	30	92
	2	1	0.1	30	40	96
	2	1	1	50	50	95
	5	-	-	80	70	95
	5	0.05	0.05	100	50	98
15	1	Applied 7/11/94. Evaluated 8/20/94.				
	2	Applied 7/12/94. Evaluated 7/25/94.				
	3	Applied 7/13/94. Evaluated 8/3/94.				

Table 8. Response to two rate of cysteine of white clover (*Trifolium repens*) in combination with ammonium nitrate and trichlopyr.

25	lb/A			% Dead		
	Trichlopyr	Ammonium nitrate	Cysteine	Applied 7/20/94, Evaluated 8/1/94	Applied 7/20/94, Evaluated 8/3/94	Applied 7/25/94, Evaluated 8/20/94
30	2	-	0.05	45	45	35
	2	1	1	50	45	40
	2	1	-	30	30	40
30	5	-	-	50	50	35
				Average		

Table 9. Response of three resistant weed species to two rates of cysteine in combination with ammonium nitrate and trichlopyr.

35	lb/A			% Dead		
	Trichlopyr	Ammonium nitrate	Cysteine	Canada thistle <sup>1</sup>	Common milkweed <sup>2</sup>	Chicory <sup>3</sup>
40	2	-	-	40	45	60
	2	1	0.05	50	60	80
	2	1	1	40	20	80
	1	-	-	60	50	80

<sup>1</sup> *Cirsium arvense*. Applied 7/26/94. Evaluated 8/20/94.<sup>2</sup> *Asclepias syriaca*. Applied 7/27/94. Evaluated 8/20/94.<sup>3</sup> *Chicorium intybus*. Applied 7/28/94. Evaluated 9/12/94.

Table 10. Weed control by 2 lb/A trichlopyr with or without ammonium nitrate or cysteine on wild carrot (*Daucus carota*), Canada thistle (*Cirsium arvense*), whorled milkweed (*Asclepias verticillata*), common milkweed (*Asclepias syriaca*), field bindweed (*Convolvulus arvensis*), chicory (*Chichorium intybus*), white clover (*Trifolium repens*), red clover (*Trifolium pratense*) aster (*Aster* spp.) and goldenrod (*Solidago* spp.).

5	2 lb/A Trichlopyr plus										% Dead	
	Ammonium nitrate	Cysteine	Wild carrot	Canada thistle	Milkweed whorled	Milkweed common	Field bindweed	Chicory	Clover, white	Clover, Red	Aster/goldenrod	Average all species
10	0	0	30 <sup>1</sup> 30 <sup>1</sup>	30 <sup>3</sup> 50 <sup>4</sup>	0 <sup>13</sup>	20 <sup>4</sup> 20 <sup>12</sup>	100 <sup>4</sup>	20 <sup>6</sup> 10 <sup>11</sup>	20 <sup>9</sup> 15 <sup>10</sup>	30 <sup>10</sup> 30 <sup>12</sup>	20 <sup>15</sup>	30
	1	0	30 30	40 80	0	40 20	100	30 10	30 30	40 30	20	35
	0	1	35 30	30 70	20	55 30	100	30 20	35 20	40 40	20	40
	0	1	40 40	50 65	20*	80 100	100	45 25	40 25	50 50	25**	53
15	1	-	40 40	60 80	30*	100 100	100	50 30	30 30	50 50	30**	53
	5 lb/A trichlopyr											
	1											
	2											
20	3											
	4											
	5											
	6											
25	7											
	8											
	9											
	10											
30	11											
	12											
	13											
	14											

<sup>1</sup> Applied 7/29/94. Evaluated 8/20/94. <sup>2</sup> Applied 8/1/94. Evaluated 8/20/94.

<sup>3</sup> Applied 8/3/94. Evaluated 8/20/94. <sup>4</sup> Applied 8/3/94. Evaluated 9/8/94.

<sup>5</sup> Applied 8/4/94. Evaluated 8/16/94. <sup>6</sup> Applied 8/6/94. Evaluated 8/20/94.

<sup>7</sup> Applied 8/8/94. Evaluated 8/20/94. <sup>8</sup> Applied 8/9/94. Evaluated 9/12/94.

<sup>9</sup> Applied 8/10/94. Evaluated 8/20/94. <sup>10</sup> Applied 8/11/94. Evaluated 9/3/94.

<sup>11</sup> Applied 8/15/94. Evaluated 9/3/94. <sup>12</sup> Applied 8/17/94. Evaluated 9/3/94.

<sup>13</sup> Applied 8/19/94. Evaluated 9/3/94. <sup>14</sup> Applied 9/22/94. Evaluated 9/3/94.

<sup>15</sup> Applied 8/24/94. Evaluated 10/9/94. <sup>\*</sup> 100% dead 9/14/94.

<sup>\*\*</sup> Resistant, not killed.



In addition, a series of date and rate tests were conducted between August 9, 1994 and August 25, 1994. A mixture of 2 lb/A trichlopyr, 1 lb/A ammonium nitrate and 0.05 lb/A cysteine was applied at 1/2 X, 1X, 1.5X, 2X, 2.5X and 3X on August 9, August 11, August 16, August 18, August 23 and August 25. Species  
5 treated included common milkweed (*Asclepias syriaca*), field bindweed (*Convolvulus arvensis*), ground cherry (*Physalis heterophylla*), dogbane (*Apocynum cannabinum*), wild carrot (*Daucus carota*), red clover (*Trifolium pratense*), whorled milkweed (*Asclepius verticillata*), canada thistle (*Cirsium arvense*), evening primrose (*Oenothera biennis*), wild lettuce (*Lactuca canadensis*), chicory (*Chicorium intybus*),  
10 dandelion (*Taraxacum officinale*), buckthorn plantain (*Plantago lanceolata*), common plantain (*Plantago major*), creeping charlie (*Glechoma hederacea*) and poison ivy (*Rhus radicans*). Complete control of all species was attained at the 2X rate of application (4 lb/A trichlopyr plus 2 lb/A ammonium nitrate plus 0.1 lb/A cysteine).

15 It is evident from the above results that the use of thiol compounds in conjunction with auxin like compounds as herbicides greatly enhances the efficiency of cytotoxicity of the herbicide. Substantially lower rates of application can be employed than previously employed to obtain substantially complete eradication of weeds and other undesired vegetation.

## 20 II. Use of Thiol Compounds as Root Formation Enhancers

The following study is directed to rooting of microcuttings produced from callus, using auxin shock, followed by treatment with a thiol compound of *malus*, *Daphne* and *Dipladnia*.

### 25 Materials and Methods

Rooting of microcuttings produced from callus of *Malus domestica* Borkh., *Dipladenia* and *Daphne odorata variegata* was studied. All three species were rooted using the same technique. Shoots (2 cm) were harvested at the end of the  
30 multiplication medium and were placed for 1 h (*Dipladenia*) or 2 h (*Malus* and *Daphne*) on a highly concentrated agarised solution of IAA at 1 mM (*Dipladenia* and *Daphne*) or 2 mM IAA (*Malus*). After this auxin treatment, the micro cuttings

were transferred to a hormone-free medium, containing the thiol compound, dithiothreitol, at different molarities, to allow root formation to proceed.

The plants were first placed in the dark for 6 days at 26°C and then transferred to the culture room. The auxin (IAA) was solubilized in 70% alcohol and the pH adjusted to 5.5. The auxin treatment took place in plastic jars and rooting was in glass tubes closed with Magenta caps. The actual multiplication and root expression media varied according to the species involved.

1) *Malus*: For the trials, we used two apple rootstocks (Jork 9 and Cepiland) which were cultured *in vitro*. The proliferation phase took place on a Murashige and Skoog (1962) (Need citation) mineral solution with 100 mg/l inositol, 1 mg/l thiamine-HCL, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCL, the plant growth regulators benzyladenine (1 mg/l) and indole-butyric acid (0.1 mg/l), and sucrose (30 g/l) (MSO medium). The pH was adjusted to 5.5. The culture conditions were: temperature 20-22°C, photoperiod 16 h and light (mol/m<sup>2</sup>/s). To further propagate the plants, 0.5 cm apical shoots were excised and transferred to a fresh medium. After 6 weeks, tufts of shoots between 1 to 3 cm in length were formed.

2) *Daphne*: An *in vitro* culture of *Daphne odorata variegata* was used. The multiplication medium was a Woody Plant Medium (WPMO) mineral solution (Lloyd and McCown 1981) (Need citation) supplemented with vitamins (Mullin et al. 1974) (Need citation) and amino acids (Skoog 1944) (Need citation). The plant growth regulators were benzylaminopurine 0.5 mg/l and  $\alpha$ -naphthylacetic acid 0.01 mg/l. Sucrose and Difco bactoagar were added at 20 g/l and 9 g/l respectively, after adjusting the pH to 5.5.

3) *Dipladenia*: An *in vitro* culture of *Dipladenia* was used. The multiplication medium was based on Murashige and Skoog (1962) mineral, vitamin and amino acid solutions supplemented with 4 mg/l 2iP (2-isopentyladenine) and 0.2 mg/l indolebutyric acid. Sucrose and agar (Sigma 9915) at 30 g/l and 7 g/l respectively were added after adjusting the pH to 5.7. The root expression media were identical to the multiplication media but were hormone free (i.e. a MSO medium for *Malus* and *Dipladenia*, and a WPMO medium for *Daphne*).

The auxin shock utilized indole-3-acetic acid (IAA). The short treatment

with auxin was a 2 h dip of the base of the cutting (2-3 mm) in an agarised solution of 1 or 2 mM IAA. The shoots were then transferred (rinsed) directly to agar medium for rooting.

With *Malus*, to investigate the timing of dithiothreitol action on rooting, one experiment was with different incubation timings. One application was during the induction phase (during 5 days following the auxin shock), then the cuttings were transferred to MSO medium. Another application was during the expression phase (after 5 days on MSO medium), then the cuttings were transferred to the medium with various DTT concentrations (day 5 to day 21).

In woody plant experiments, each treatment consisted of 22 or 33 cuttings. Experiments were repeated 2 times. Rooting was measured as the number of roots per cutting after 2 and 3 weeks following the auxin shock for *Malus domestica* var Jork 9 (easy to root) and *Malus domestica* var Cepiland (difficult to root) and 3 and 4 weeks following the auxin shock for *Daphne odora* (difficult to root) and 4, 6, and 8 weeks for *Dipladenia* (difficult to root).

Statistical data are expressed using GBStat software one, two or three way analysis of variance and means comparison by Dunnett Test at 0.05. The test compares the control treatment (DTT = 0). Data are presented as the probability level for each treatment and as the results of the Dunnett's Test.

## Results

### *Daphne odora*

*Daphne odorata* has always been considered as a difficult-to-root species. In our experiments, rooting was greatly improved by using DTT at low concentration (0.1 to 0.25 mM) (Table 10) after 4 weeks following auxin shock.

Another aspect of using DTT is the absence of callus formation at the base of the microcuttings. Large callus are always developing in multiplication medium of rooting without DTT but not in its presence.

### *Dipladenia*

In these experiments, cuttings were obtained either from apical shoot segments (apex) or from mononodal shoot segments. After 8 weeks, the rooting was highly improved using apex cuttings, and after auxin shock, incorporating DTT in the rooting medium at concentrations of 0.1 or 0.25 mM (Table 11). As in the

*Daphne* experiments, we also noticed the absence of callus formation in plants treated with DTT.

*Malus domestica* var Jork 9

With this "easy-to-root" rootstock strain, use of DTT consistently promoted adventitious rooting. Best results were at concentrations of 0.35 and 0.3 mM of DTT (Table 12). After 3 weeks, the cuttings without auxin shock did not give a significant number of roots either in the presence or in the absence of DTT.

*Malus domestica* var Cepiland

This strain of *Malus* rootstock is more difficult to root than Jork 9. At 3 weeks after auxin shock, the application of DTT under the same conditions as Jork 9 always gave results significantly better than the controls without DTT. The absence of an auxin shock did not induce a significant number of roots (Table 13). Higher concentrations of DTT compared with Jork 9 were required to give the best results in the preparation of plants producing roots and in the mean number of roots/plants.

Table 11. Rooting of *Daphne odora* after auxin shock in the presence of varying concentrations of DTT. Data are expressed as % of rooting and number of roots and mean comparison by Dunnett Test at 0.05. Means with the same letter are not different from the control ( $p < 0.001$ ).

DTT, mM	Rooting, %	Roots/plant
0.00 (control)	18 <sup>a</sup>	0.21 <sup>a</sup>
0.1	63	2.70
0.25	54	1.42
0.50	30 <sup>a</sup>	0.64

Table 12. Rooting in *Dipladenia* apical cuttings. The best rooting response was with 0.25 and 0.1 mM dithiothreitol (DTT) after 8 weeks. Means with the same letter are not different from the control ( $p < 0.001$ ).

Type of cutting	Auxin shock	DTT, mM	Roots/plant	%
Apex	-	0.00	1.6 <sup>a</sup>	13 <sup>a</sup>
		0.1	1.9 <sup>a</sup>	15 <sup>a</sup>
		0.25	2.3 <sup>a</sup>	15 <sup>a</sup>
	+	0.00	3.3 <sup>a</sup>	30 <sup>a</sup>
		0.1	4.3 <sup>a</sup>	77
		0.25	6.5 <sup>a</sup>	89

Node	-	0.0	0.0	0
		0.1	0.8	9
		0.25	1.1	10 <sup>a</sup>
	+	0.00	0.3	2
		0.1	0.3	5
		0.25	1.1	9

Table 13. Response of cuttings of *Malus domestica* var Jork 9 3 weeks after auxin shock. Means with the same letter are not different from the control.

5

	DTT, mM	Rooting, %	Roots/plant
	0.0	73 <sup>a</sup>	3.45 <sup>a</sup>
	0.1	77 <sup>a</sup>	3.77 <sup>a</sup>
	0.15	91	3.77 <sup>a</sup>
10	0.2	82 <sup>a</sup>	4.09
	0.25	91	4.95
	0.3	96	6.14
	0.5	77 <sup>a</sup>	3.23 <sup>a</sup>
	1.0	73 <sup>a</sup>	2.55 <sup>a</sup>
15	2.0	23	0.23

Table 14. Effect of DTT concentration on rooting of *Malus domestica* var Cepiland cuttings. Means with the same letter are not different from the control ( $p < 0.05$ ).

	DTT, mM	Rooting, %	Roots/plant
20	0.0 (Control)	77 <sup>a</sup>	2.59 <sup>a</sup>
	0.1	73 <sup>a</sup>	1.82 <sup>a</sup>
	0.25	68 <sup>a</sup>	1.68 <sup>a</sup>
	1.0	91	4.64

In the next study, species *Glycine max*, *Phaseolus aureus* and *Malus*

25 *domestica* were studied. In addition, the thiol treatment was varied as to the time administered after the auxin shock.

#### Rooting of Shoots of Soybean and Mungbean

Seed of soybean (*Glycine max*, var Williams) or mungbean (*Phaseolus aureus*) were soaked in water for several hours, planted in moist vermiculite and  
30 germinated and grown in the greenhouse for about 2 weeks. Shortly after the

epicotyl was fully extended but before the expansion of the first trifoliate leaf, shoots were harvested by cutting just above the roots and transferred to shell vials (10 ml) containing 5 ml of aqueous rooting solution. Rooting was continued in the light (fluorescent, 200 mol/m<sup>2</sup>/s, 8 h light period) for 7 to 10 days. All roots were counted on each of 3 to 5 cuttings in 3 replications. Results are means  $\pm$  standard deviations among replicates.

#### Micropropagation and Rooting of Shoots of Apple in Culture

The plant was an apple rootstock Jork 9 obtained from B. Kunneman (COST, NL-2160 AB LISSE). The plants were already established *in vitro* before the start of the experiments.

In the proliferation phase, plants were formed in tufts of shoots varying between 1 and 3 cm in length. To multiply the plants, an apex of 0.5 cm was excised and transferred to fresh medium. Apices were cultured in glass jars containing 100 ml of medium and covered with Saran film (PVDC Dow Chemical). The multiplication medium and the culture room conditions were described in Seingre et al. (1991) Acta Hortic. 289: 151-155. The medium contained MS mineral solution (macro and micro elements) with 100 mg/l inositol, 1 mg/l thiamine HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, the plant growth regulators benzyladenine (1 mg/l) and indolebutyric acid (0.1 mg/l) and sucrose (30 g/l). The pH was 5.5. The culture conditions were: temperature, 20 - 22°C, photoperiod, 16 h and light, 15 W/m<sup>2</sup>.

For the rooting phase, shoots of 2 cm in length were harvested at the end of the proliferation stage and were given a short treatment with auxin before transfer to a rooting medium (medium identical to the multiplication medium without hormones) to initiate rooting. The sulfhydryl compounds were prepared aseptically by filtration and introduced with the concentrated molten agar rooting medium just before solidification. After transfer, the plants were kept in darkness at 26°C for 6 days and then returned to the culture room. The auxin treatment and the rooting were in glass jars. Rooting is expressed as the number of shoots per tuft producing > 5 roots. Each treatment was 22 tufts and experiments were repeated 3 times. Rooting was measured as the ratio of the number of roots produced/total number of shoots.

### Auxin Shock

The auxin shock utilized indole-3-acetic acid (IAA). The short treatment with auxin was a 2 h dip of the base of the cutting (2-3 mm) in an agarised solution of 2 mM IAA. The shoots were then transferred (rinsed) directly either to agar  
5 (apple) or distilled water in shell vials (soy- and mungbean) containing the test substances.

### RESULTS

In either the presence and in the absence of auxin, the numbers of roots were about doubled by the addition of the thiol compounds GSH or DTT compared to no auxin shock (- auxin) or the auxin shock alone (+ auxin). See Table 15. Both  
10 mungbean and soybean showed an optimum curve in the presence of auxin at 0.1 mM for glutathione and 1 mM for dithiothreitol. In the absence of auxin, rooting overall was much less and the optimum was less clear. However in the absence of the auxin shock, rooting appeared to be stimulated at approximately the same concentration as in the presence of 2,4-D especially with soybeans. Low  
15 concentrations of dithiothreitol (0.1 to 0.05 mM) appears to inhibit rooting of mungbean shoots in the presence of auxin.

The appearance of the roots for soybean shows striking differences. Roots in the presence of glutathione or dithiothreitol were not only more numerous but generally were longer than the roots of comparable control shoots treated either in  
20 the absence or presence of the auxin shock. Additionally, with dithiothreitol and glutathione, roots formed higher on the stem.

In addition to improving the quality of roots formed in the presence of the auxin shock, glutathione and dithiothreitol also improved shoot elongation following the auxin shock (Table 16). With auxin shock alone, shoot growth was reduced by  
25 approximately 50%. At optimum concentrations of glutathione and dithiothreitol for rooting, shoot growth in the presence of auxin was restored nearly to levels in the absence of auxin.

With *Malus* cuttings growing in agar, the thiol compounds were not only effective in rooting but also promoted shoot growth. As with soybean and  
30 mungbean, the compounds were effective both in the presence and in the absence of an auxin shock. See Table 17.

Table 15. Rooting of soybean shoots promoted by glutathione and dithiothreitol in the presence and absence of auxin shock (2mM IAA, 2h)

Results are means of ten shoots in each of 3 replicate trials  $\pm$  standard deviations among trials.

5

Treatment	Roots per shoot	
	- Auxin	+ Auxin (2mM)
None	15 $\pm$ 3	28 $\pm$ 1
Glutathione (0.25 mM)	22 $\pm$ 2	45 $\pm$ 6
Dithiothreitol (0.5 mM)	21 $\pm$ 4	40 $\pm$ 5

10

Table 16. Shoot length of soybean promoted by glutathione and dithiothreitol in the presence and absence of auxin shock (2 mM IAA, 2h).

Results are means of ten shoots in each of 3 replicate trials  $\pm$  standard deviations among trials.

15

Treatment	Shoot length cm	
	- Auxin	+ Auxin (2 mM)
None	10 $\pm$ 2	5 $\pm$ 1.5
Glutathione (0.1 mM)	12 $\pm$ 3	12 $\pm$ 2
Dithiothreitol (1 mM)	7 $\pm$ 1	9 $\pm$ 1

20

Table 17. Rooting of soybean promoted by dithiothreitol and effects on root length. Results are means of three replicates of 10 shoots each  $\pm$  standard deviation among replicates.

25

Treatment	Dithiothreitol	Roots	
		Number/cutting	Length (cm)
Control (water only)	None	13 $\pm$ 1	2.5 $\pm$ 0.5
Auxin shock	None	19 $\pm$ 3	0.45 $\pm$ 0.05
Auxin shock	0.1 mM	40 $\pm$ 10	4.1 $\pm$ 0.5
Auxin shock	0.3 mM	53 $\pm$ 13	3.0 $\pm$ 0.4

30

Table 18. Response of rooting of *Malus* var Jork 9 to glutathione (GSH), 3 weeks after auxin shock.

35

Means followed by the same letter are not different from controls ( $p > 0.001$ ).



	GSH	Rooting, %	Roots/Plant, number
	0.0 (Control)	73 <sup>a</sup>	3.45 <sup>a</sup>
	0.1	77 <sup>a</sup>	3.77 <sup>a</sup>
	0.15	91	3.77 <sup>a</sup>
5	0.2	82 <sup>a</sup>	4.09
	0.25	91	4.95
	0.3	96	6.14
	0.5	77 <sup>a</sup>	3.23 <sup>a</sup>
	1.0	73 <sup>a</sup>	2.55 <sup>a</sup>
10	2.0	23	0.23

Table 19. Rooting of *Hedera* cuttings after 1 month promoted by dithiothreitol. Results are mean of replicated determinations of 6 to 9 cuttings/treatment  $\pm$  mean averages deviations between the means.

	Treatment	Roots/cutting	Cuttings with no Roots, %
	Control (water only)	2 $\pm$ 1	33
	Auxin shock	5 $\pm$ 2	30
20	Auxin shock + 0.1 mM dithiothreitol	13 $\pm$ 9	10
	Auxin shock + 0.3 mM dithiothreitol	13 $\pm$ 0	0

#### Alternative Rooting Procedure

The initial step can be reached by a one day auxin shock with indolebutyric acid (25  $\mu$ M) on stem discs or 2 h IAA shock (1 mM) on shoots. Both treatments are enough for induced formation of roots in *Malus Jork 9*. Root induction is obtained in the 4-5 days following the auxin shock, root elongation of expression needing 3 to 5 days more. The time of DTT application in the subsequent steps give results that show promoting effects of DTT. In one experiment, with application of DTT at the time of induction or at the time of expression, preliminary results showed that action of DTT was similar for the induction phase as well as the expression phase.

In herbaceous plants, easy-to-root, the response to DTT and GSH is nearly double the amount of roots produced both in the presence or absence of auxin treatment. With woody plants, which are more difficult to root than herbaceous, the best response is given with only the DTT in the presence of auxin or in auxin shock.

5 III. Effect of Thiol Compounds on Cytotoxicity of Anticancer Compounds With Cancer Cells

The use of cysteine in combination with an antitumor sulfonylurea LY181984 (N-(p-tolylsulfonyl),N-(p chlorophenyl) urea. The compositions were tested employing HeLa cells in culture. Mammalian cells were grown in 175 cm<sup>2</sup> flasks in  
10 minimum essential medium at 37°C with 10% bovine calf serum (heat inactivated). Cell proliferation was determined by direct cell counts using a hemacytometer. Cysteine at a concentration of 100µM was without effect on growth of HeLa cells, but in the presence of 100µM LY181984 was effective in preventing HeLa cell growth. Cysteine was effective in enhancing the activity of 100 µM LY181984 over  
15 the concentration range of 10 to 1000 µM (all concentrations equally effective) (Table 19). Concentrations lower than 10 µM were not tested.

With longer periods of growth the combination of LY181984 plus cysteine began to result in programmed cell death (apoptosis) of the treated cells (Table 18). Overall, the effect of the cysteine was to increase the effectiveness of LY181984 by  
20 approximately two log orders, i.e. approximately 100 times less LY181984 was required to kill the cells in the presence of 100 µM cysteine, than in its absence.

Table 20. Response of HeLa cells to LY181984 and effect of varying concentrations of L-cysteine.

25

LY181984	L-cysteine	cells/mm <sup>2</sup>
None	None	933
100 µM	None	408
	10 µM	248
	100 µM	268
	1000 µM	242

30

Table 21. Cell number after 168 of HeLa cells treated with 100  $\mu$ M LY181894 in the presence or absence of 100  $\mu$ M L-cysteine.

	LY181894	L-cysteine	cells/mm <sup>2</sup> *
5	None	None	1987 $\pm$ 21
		100 $\mu$ M	2066 $\pm$ 36
	100 $\mu$ M	100 $\mu$ M	395 $\pm$ 57

10 \* Initial cell number 150 cells/mm<sup>2</sup> between 72 and 168 h. The number of control cells increased 3.3-fold whereas those treated with cysteine plus LY181894 decreased by 10%. Remaining cells were largely apoptotic.

It is evident from the above results, that the addition of thiol compounds in conjunction with a variety of cell modulating agents can greatly enhance the effectiveness of the cell modulating agent. Thus, with compounds which encourage cell proliferation such as auxins, where the auxin can serve as a herbicide or rooting formulation, thiol compounds are found to enhance the effectiveness of the auxin for its cell modulating effect. With chemotherapeutic agents, particularly those associated with inhibition of a surface membrane NADH oxidase, the thiol compounds substantially enhance the cytotoxic effect, permitting substantially lower concentrations of the cytotoxic agent, while retaining the desired cytotoxic effect.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. In a method for inhibiting cellular growth of undesired cells using a cytotoxic agent, by contacting said cells with said cytotoxic agent under cytotoxic conditions, the improvement which comprises:
  - 5 contacting said cells in conjunction with said cytotoxic agent, with a thiol compound in an amount sufficient to enhance the cytotoxic effect of said cytotoxic agent, where said cytotoxic agent is administered in an amount less than the amount required to obtain cytotoxicity in the absence of said thiol compound.
- 10 2. A method according to Claim 1, wherein said cells are plant cells as part of a growing plant and said cytotoxic agent is an auxin-type herbicide.
  3. A method according to Claim 2, wherein said contacting is in conjunction with ammonium nitrate as an adjuvant in an amount sufficient to  
15 enhance cytotoxicity.
  4. A method according to Claim 2, wherein said thiol compound is cysteine, dithiothreitol or glutathione.
  - 20 5. A method according to Claim 2, wherein said herbicide and thiol compound are administered as a spray.
  6. A method according to Claim 1, wherein said cells are mammalian cells and said cytotoxic agent is a chemotherapeutic agent.  
25
  7. A method according to Claim 6, wherein said mammalian cells are in culture and said cytotoxic agent is a sulfonylurea.
  8. In a method for inhibiting the growth of plants using an auxin-type  
30 herbicide, by contacting said plants with said herbicide under herbicidal conditions, the improvement which comprises:
    - contacting said plants in conjunction with said herbicide, with a thiol

compound in an amount sufficient to enhance the herbicidal effect of said herbicide, where said herbicide is administered in an amount less than the amount required to obtain the same level of plant death in the absence of said thiol compound.

5           9.     A method according to Claim 8, wherein said herbicide is 2,4-dichlorophenoxyacetic acid or trichlopyr, and is administered at a rate of about 1 to 6 pounds per acre, and said thiol compound is administered at a rate of about 0.01 to 2 pounds per acre.

10          10.    A method according to Claim 9, wherein said contacting comprises contacting with from about 0.025 to 2 pounds per acre.

11.     In a method for inhibiting cellular growth of mammalian tumor cells in the presence of normal cells using a cytotoxic agent, by contacting said cells with  
15   said cytotoxic agent under cytotoxic conditions, the improvement which comprises:  
          contacting said mammalian tumor cells in conjunction with said cytotoxic agent, with a physiologically acceptable thiol compound in an amount sufficient to enhance the cytotoxic effect of said cytotoxic agent, where said cytotoxic agent is administered in an amount less than the amount required to obtain cytotoxicity in the  
20   absence of said thiol compound.

12.     A method according to Claim 11, wherein said thiol compound is cysteine or glutathione and said cytotoxic agent is a chemotherapeutic agent.

25          13.    A method according to Claim 12, wherein said chemotherapeutic agent is a sulfonylurea.

14.     In a method for inducing rooting by subjecting shoots to auxin shock or growth in auxins, followed by rooting in a rooting medium, the improvement  
30   which comprises:  
          rooting said shoots in rooting medium comprising a rooting enhancing amount of a thiol compound.

15. A method according to Claim 14, wherein said shoots are subjected to auxin shock with an auxin at a concentration in the range of about 0.5 to 5 mM and said shoots are then rooted in a rooting medium comprising a thiol compound for at least one day in the next 10 days.

5

16. A method according to Claim 15, wherein said thiol compound is cysteine, glutathione or dithiothreitol.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02703

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 39/04, 43/40

US CL : 504/254, 323

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 504/254, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS: triclopyr (and synonyms), cystein-, synerg-, glutathion-, dithiothreitol, thiolpropion-

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,169,720 (SCHACHT ET AL.) 02 October 1979, column 6, line 9 through column 7, line 28; column 19, lines 10-35.	2-5, 8-10
Y	US, A, 4,396,418 (SCHIRMER ET AL.) 02 August 1983, column 9, lines 39-52; column 15, lines 55-56.	2-5, 8-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* *A* *E* *L* *O* *P*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	*T* *X* *Y* *&*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search

30 MAY 1996

Date of mailing of the international search report

10 JUN 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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Authorized officer

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02703

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
2-5, 8-10

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02703

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

- I. Claims 2-5 and 8-10, drawn to herbicidal methods comprising application of a cytotoxic compound with a synergistic thiol compound, classified in Class 504, subclass 130+. Species are as follows:
  - A. 2,4-D (504/145)
  - B. Trichlopyr (504/130)
- II. Claims 14-16, drawn to methods to induce rooting in plants, classified in Class 504/ subclass 320+. Species are as follows:
  - A. Cysteine, glutathione, thiolpropionic acid (504/320)
  - B. Dithiothreitol (504/349)
- III. Claims 6, 7, and 11-13, drawn to methods of treating mammals or mammalian tumors, classified in Classes 424 or 514. Species are as follows:
  - A. Chlorambucil, melphalan (chloroethylaminophenylalkanoic acids: 514/561+)
  - B. Busulfan (butanediol dimethanesulfonate esters: 514/711)
  - C. Carmustine, lomustine (N-chloroethyl-N-nitrosoureas: 514/589)
  - D. Streptozocin (hexose containing nitrosoureas: 514/459)
  - E. Thiotepe (+sulfotepe?) (-P(=S)-O-P(=S)-: 514/106)
  - F. Dacarbazine (1,3-diazoles: 514/385)
  - G. Methotrexate (polycyclic 1,4-diazines: 514/249)
  - H. 5-Fluorouracil, Cytarabine (1,3-diazines: 514/274)
  - I. Azauridine (asymmetrical triazines: 514/242)
  - J. Mercaptopurine, thioguanine (purines: 514/262)
  - K. Vinblastine, vincristine (vinca alkaloids: 514/283)
  - L. Actinomycin D (daetinomycin, cyclopeptides: 514/9)
  - M. Adriamycin (alicyclic ketones: 514/691)
  - N. Bleomycins (glycopeptides: 514/8)
  - O. Mithramycin (plicamycin; hexoses: 514/460)
  - P. Mitomycins (5-membered hetero ring with one nitrogen atom: 514/411)
  - Q. Asparaginase (peptides: 514/2+)
  - R. Cisplatin (inorganic Pt containing: 424/649)
  - S. Sulfonylureas (514/592)
  - T. Capsaicin (carboxamides: 514/627).

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the claims are drawn to three distinct methods of use.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as indicated above within each Group. The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: PCT Rules 13.1 and 13.2 do not provide for multiple products. The species are drawn to herbicidal/pharmaceutical agents which are diverse both structurally and functionally.

Claim I is generic to Groups I and II.

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